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Use of an Agent to Reduce Scattering in Skin

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Background and Objective: **A method to increase light transport deeply into target areas of tissue would enhance both therapeutic and diagnostic laser applications. The effects of a hyperosmotic agent on the scattering properties of rat and hamster skin were investigated.**
Study Design/Materials and Methods: **A hyperosmotic agent, glycerol, was applied in vitro and in vivo to rat and hamster skin to assess the changes in tissue optical properties. Changes in the reduced scattering coefficient after application of the agent in vitro to rat skin and after the skin has been rehydrated were assessed to evaluate the effect of the agent on tissue.**
Results: **Experimental results showed a transient change in the optical properties of in vitro rat skin. A 50% increase in transmittance and decrease in diffuse reflectance occurred within 5–10 min after the introduction of anhydrous glycerol. In addition, reduction of light scattering with this technique increased depth of visibility with optical coherence tomography. Injection of glycerol under the skin allowed in vivo visualization of blood vessels.**
Conclusions: **The application of the agent reduces the amount of refractive mismatch found in the tissue and markedly reduces random scattering, thereby making the skin less turbid for visible wavelengths for a controlled period of time. Lasers Surg. Med. 24:133–141, 1999.** **© 1999 Wiley-Liss, Inc.**

Key words: controlled optical properties; light scattering; optical coherence tomography (OCT); glycerol; index-matching agent; hyperosmotic

INTRODUCTION

The complex morphological nature of tissue provides a highly scattering medium for visible and near-infrared wavelengths. Much of the light scattering in biological tissues is due to variations in polarizability, which can be characterized by variations in the index of refraction. In addition, the shape, size, and distribution of tissue constituents play an important role in the overall scattering properties of tissue. Skin is a highly complex tissue, with many inhomogeneities. The principal cell type of the epidermis is the keratinocyte, but it also contains melanocytes and Langerhans cells [1]. The dermis consists mainly of a network of collagen fibers, elastic fibers, and an interfibrillar ground substance consisting of glycosaminoproteoglycans, salts, and water, in addition to fibroblasts, the principle cells of the dermis.

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The variations in refractive index cause much of the random scattering within the dermis, which decreases light penetration into tissue. Skin components have a range of refractive indices, most of which are different from that of the interstitial space. For example, melanin has a refractive index of about 1.7 [2], fully hydrated collagen has a value of 1.43 [3], adipose tissue has a value of 1.46 [4], and the nucleus has a value of 1.36 [5,6].

Light-based therapeutic and diagnostic techniques could be improved if scattering within tissue were temporarily reduced. Laser surgery and therapy would benefit from the increased depth of penetration. Imaging modalities, such as fluorescence microscopy, confocal imaging, and optical coherence tomography (OCT), would also benefit from an increased penetration depth.

The aim of the present study is to demonstrate a technique using a chemical agent to alter the optical properties of skin. A number of studies have described altering the optical properties of tissue by means of physical or chemical manipulation. Among these are compression [7], coagulation, and dehydration, which causes the packing of cellular components to reduce reflection/refraction due to the cellular-interstitial space interface [8–10]. In addition, it is well established that immersion into index-matching fluids greatly reduces the amount of specular scattering of a dispersive system. Previous studies have demonstrated that osmotically active chemical agents can be used to alter the optical properties of sclera [11–15]. Studies on phantoms that simulate tissue have also shown changes in scattering properties induced by glucose [16,17].

The present study illustrates the effects that a hyperosmotic agent has on the optical properties of skin. Data are presented on the degree of transparency that occurs with the application of an osmotic agent to the skin of Sprague-Dawley rats and Syrian Golden hamsters. Although a number of agents could fulfill the requirements for the study, such as glucose solution, dimethyl sulfoxide solution, or Trazograph, glycerol is the agent of choice in the present study. It is biologically inert and is widely used in cosmetics and medicine [18,19]. In addition, it has a refractive index of about 1.47 [20], which is similar to that of collagen, which makes up 77% of the dry weight of skin [1].

We hypothesized that the agent reduced random scattering primarily by localized dehydration and better index matching with collagen. This hypothesis was supported by changes in the optical properties of in vitro and in vivo rat and hamster skin with the application of glycerol. A demonstration of the ability of the method to increase depth of visibility in OCT is presented.

MATERIALS AND METHODS

Sprague-Dawley rats and Syrian Golden Hamsters weighing approximately 250 g and 150 g, respectively, were used for the study. The animals were anesthetized with a 3:4 solution of xylazine:ketamine (0.1 ml/kg body weight) prior to all procedures.

Photographs of Tissue Clearing

The skin of Sprague-Dawley rats was harvested, and the underlying muscle layer was removed after the rats were euthanized. The freshly excised skin (thickness of 1.45 ± 0.03 mm) was placed over a resolution target (Fig. 1a) and exposed to anhydrous glycerol for different periods of time; photographs were taken through a surgical microscope (Wild Leitz M650).

For the in vivo study, animals were anesthetized with a 3:4 mixture of xylazine:ketamine (0.1 ml/kg body weight). Photographs demonstrating the effect of anhydrous glycerol on the in vivo hamster skin were taken. The glycerol (0.1 ml) was injected under the skin and observed for 20 min.

Transmission and Diffuse Reflectance Measurements

Optical property measurements were performed on in vitro samples of rat dorsal skin. Spectrophotometer measurements were made with a Varian Cary 5E spectrophotometer capable of scanning wavelengths of 175–1,800 nm. A single integrating sphere accessory (Varian 00 100449 00) was used to collect the amount of collimated light transmitted by and diffusely reflected by the skin. Native skin samples, which had not been treated with any chemical agents, were used as controls. Relative transmission and diffuse reflectance were measured for native skin samples and samples treated with glycerol placed between two glass slides. Treatment with glycerol consisted of applying the agent to the dermal side of the skin samples for periods of 5, 10, and 20 min. To demonstrate that the treatment was reversible, samples were hydrated with physiologic phosphate buffered saline solution for 20 min. Optical properties for each case were then calculated with the inverse adding-doubling method [21],

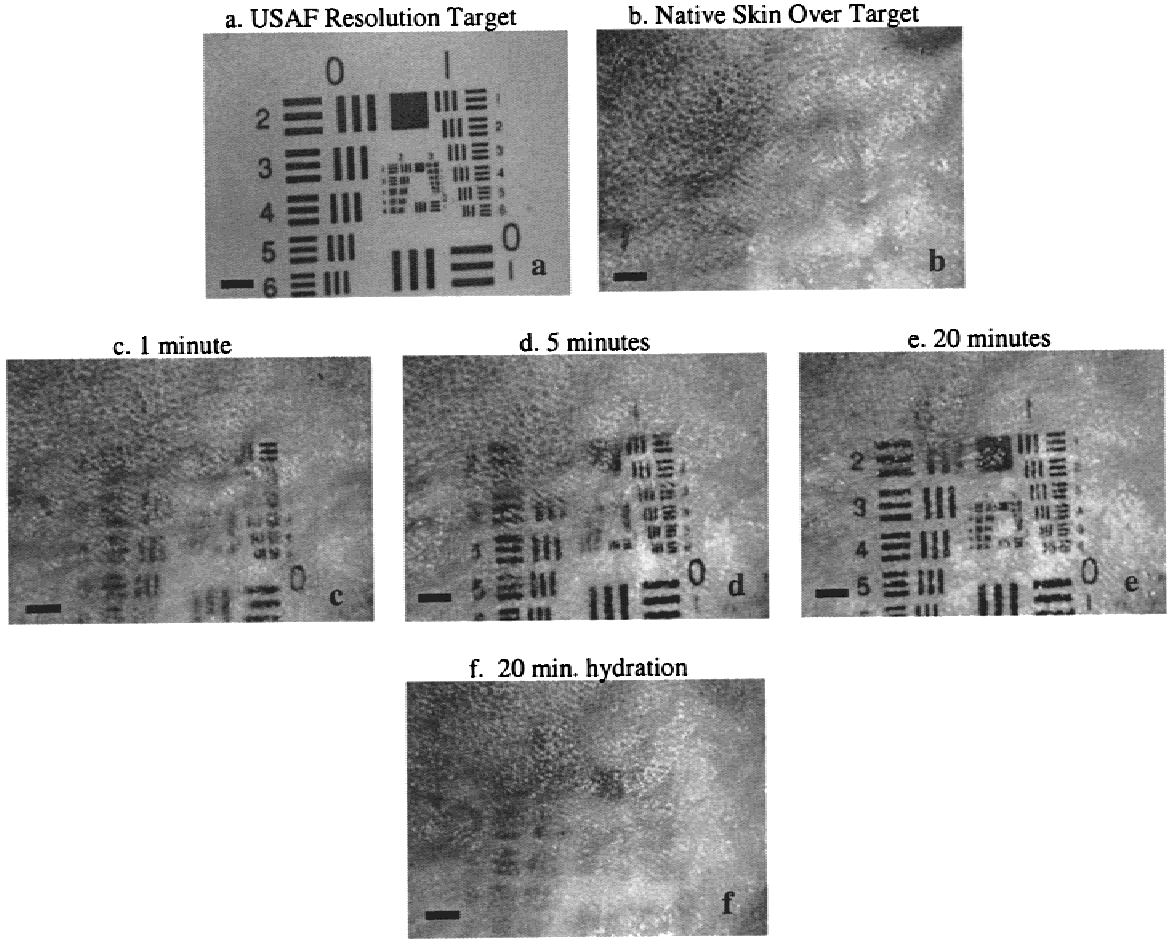

Fig. 1. Visual changes in the turbid nature of in vitro rat skin with the application of glycerol to the subdermal side. **a:** USAF resolution target used under skin samples. **b:** Native skin placed over the resolution target. **c:** One-minute application of glycerol. **d:** Five-minute application of glycerol. **e:** Twenty-minute application of glycerol. **f:** Twenty-minute rehydration with phosphate buffered saline solution. Bar = 0.5 mm.

which accounted for multiple reflections at the air–glass and glass–tissue interfaces.

Assuming a relationship of the type:

$$\mu_s' = C\lambda^{-n} \quad (1)$$

$$\ln \mu_s' = C - n \ln \lambda \quad (2)$$

Least squares values of n were computed (range = 500–1,200 nm) for the tissue samples treated with the osmolyte for different periods of time.

OCT

The effect of glycerol on in vitro hamster skin was also investigated by OCT. The OCT system, previously described [22], incorporates a 1,280-nm center wavelength superluminescent diode in a Michelson interferometer. OCT images were acquired of a dark brown human scalp hair (75 ± 3 μm diameter) placed underneath newly excised hamster skin. The skin was then soaked in glycerol for 10 min and replaced on top of the hair for the acquisition of a new set of images.

RESULTS

In vitro and in vivo experiments demonstrated the increase in light transmission through tissue with the application of glycerol. Figure 1 illustrates the dynamic changes in skin turbidity after application of glycerol in terms of the visibility of a USAF resolution target that was placed under a skin sample treated with glycerol for different periods of time. A photograph of the USAF resolution target is presented in Figure 1a. Figure 1b shows a freshly harvested rat skin specimen

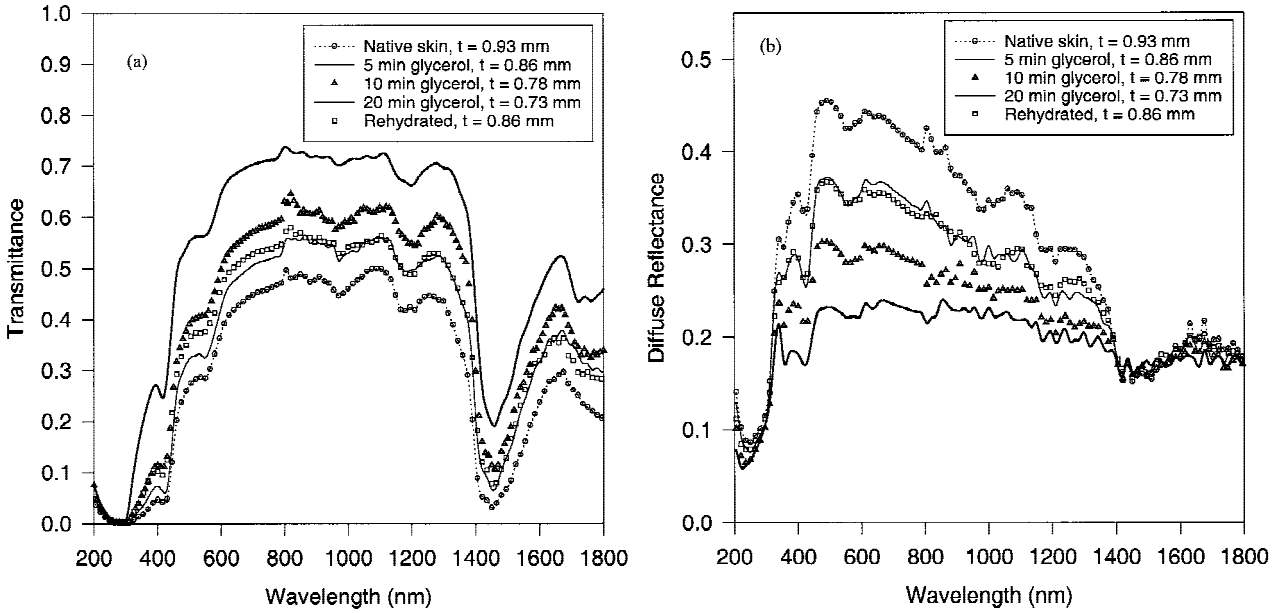

Fig. 2. Measured optical changes for in vitro rat skin before and after application of glycerol. Shown are values for native skin, application of glycerol for 5, 10, and 20 min, and rehydration of the skin with phosphate buffered saline solution. The thickness of the sample after each step is also given (t). **a:** The plot shows the increase in light transmittance due to glycerol and subsequent decrease with rehydration. **b:** The plot shows the decrease in diffuse reflectance with time in glycerol and subsequent increase with rehydration.

(thickness of 1.45 mm) placed directly over the resolution target. One to five minutes after applying glycerol to the dermal side of the skin, the resolution target became increasingly visible through the skin (Fig. 1c,d). After 20 min, the features of the target were seen through the skin specimen (Fig. 1e). To demonstrate the reversible nature of this method, the glycerol soaked specimen was rehydrated with phosphate buffered saline solution; after 20 min of hydration (Fig. 1f), the turbidity of the skin specimen returned, and the target was no longer clearly visible.

Optical property measurement results confirmed the visually observed reduction in scattering. Figure 2a,b shows the increase in transmittance and decrease in reflectance as a function of time the skin was soaked in glycerol. Rehydration of the skin sample for 20 min almost restored the native optical properties of the skin. Transmittance and reflectance after rehydration were similar to values obtained when the native tissue was placed in glycerol for 5 min. The associated reduced scattering coefficient, μ_s' (Fig. 3), and the absorption coefficient, μ_a (Fig. 4), are shown for the wavelength range of 300–1,300 nm. The skin-reduced scattering coefficient in the visible range dramatically decreased with exposure to glycerol. The thickness of the tissue at each stage is given in the captions to Figures 2 and 3. There was a

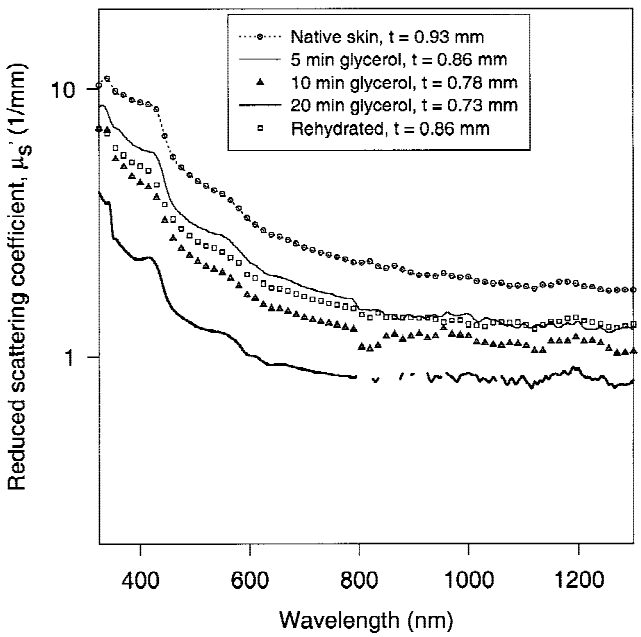

Fig. 3. Calculated reduced scattering coefficient, μ_s', for in vitro rat skin before and after application of glycerol for 5, 10, and 20 min, and rehydration.

21.5% decrease in thickness going from the native tissue to the tissue treated with glycerol for 20 min. An increase occurred when the sample was rehydrated. We estimated a 60% decrease in hy-

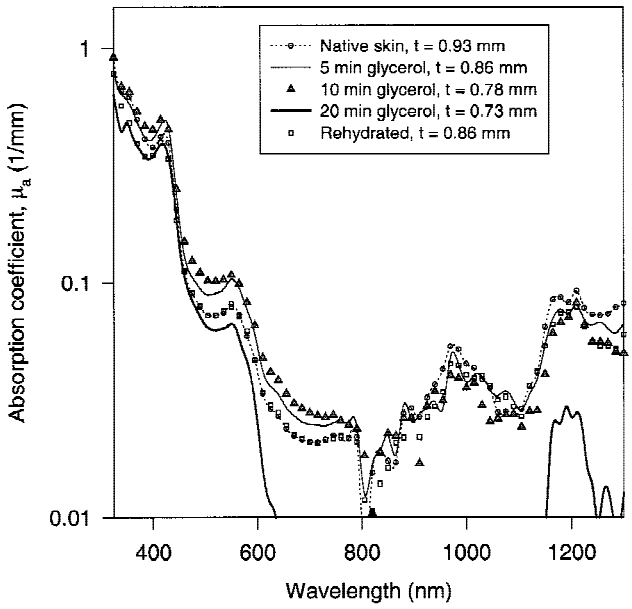

Fig. 4. Changes in the calculated absorption coefficient, μ_a, of in vitro rat skin treated with glycerol for 5, 10, and 20 min and then rehydrated with phosphate buffered saline solution.

dration, based on changes in the water absorption peaks.

Least squares computation resulted in values of n = 1.12 for the native tissue, with a subsequent decrease in n with increased time in glycerol. These values were 1.09 for 5 min, 0.85 for 10 min, 0.52 for 20 min, and 0.90 for the rehydrated sample.

An image taken of in vivo hamster skin exposed to glycerol is shown in Figure 5. The glycerol was injected under the skin (0.1 ml). We noted that the effect of tissue clearing was similar to that seen in excised skin specimens. In Figure 5, a very distinct border is seen between the areas affected by the agent and the native skin where the agent did not penetrate. A light ring can be seen where the water originally in the center of the area of interest has been displaced by the glycerol and is confined to the border. The area around the injection site that is unaffected by the glycerol remains turbid. The glycerol-injected region becomes more transparent. The muscle layer beneath the skin is visible. In addition, blood vessels not seen in the untreated tissue are seen in the area permeated with glycerol (note the diagonal vessel shown by an arrow, which is seen in the area with glycerol but disappears in the turbid native tissue).

The OCT scan of native hamster skin covering a human hair is shown in Figure 6. Different

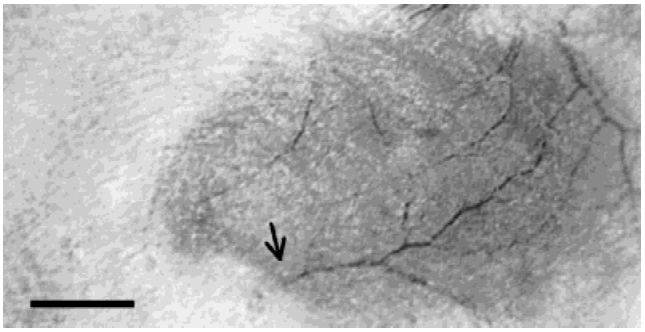

Fig. 5. Effect of glycerol on in vivo hamster skin. The glycerol was injected underneath the skin and it spread out only minimally (dark area). Vasculature not visible in the native skin is clearly seen in the area affected by glycerol. The arrow indicates a vessel that is visible on the right (area affected by glycerol) but cannot be seen in the unaffected skin area. Bar = 1 cm.

layers in the skin (epidermis, dermis, adipose tissue, cutaneous muscle, and collagenous connective tissue) can be identified in this figure. The underlying hair and tissue border are not seen because the OCT signal decreased below the noise level due to the high light attenuation in the native skin sample. The changes in the OCT scan after the skin was put in glycerol for 10 min are shown in Figure 6b. Reduced scattering produced dark areas. Most important, the underlying hair is now visible (shown as the bright region in the lower center of the image).

DISCUSSION

As a scattering medium, skin is a highly complex structure consisting of many inhomogeneities. The major constituent is collagen; however, there are substantial differences in morphology even within the different layers. Collagen bundles are tightly packed, and there is substantially less ground substance in the reticular dermis, which constitutes the bulk of the dermis. The collagen fibers in the papillary dermis are smaller (0.3–3 μm) and are more loosely distributed than in the reticular dermis (10–40 μm in diameter). Elastic fibers consisting of microfibrils, 10–12 μm diameter, embedded in an amorphous elastin matrix are interwoven with the collagen fibers.

Because of the intricate nature of the skin, it is a challenging problem to determine the major contributors to scattering. Indeed, all cellular and intracellular component types contribute to the scattering properties of skin. Because collagen makes up nearly 80% of the dry weight of skin, we

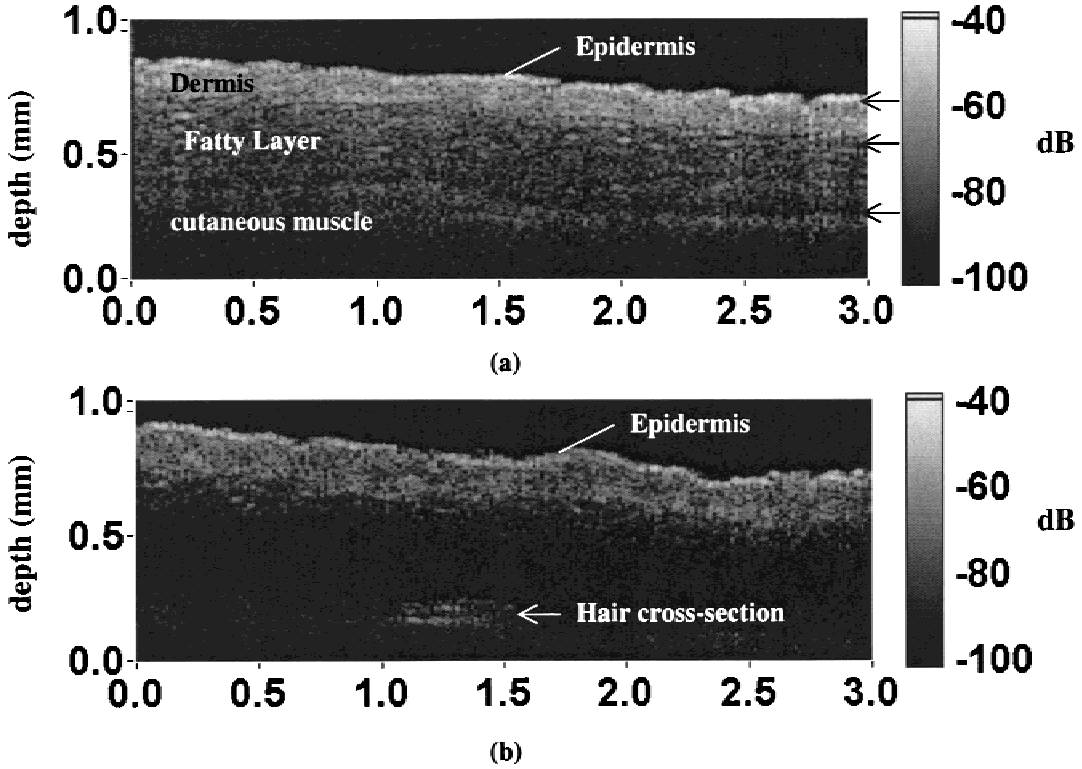

Fig. 6. Optical coherence tomographic cross-section images of in vitro hamster skin overlying human hair. **a:** The hair is not visible due to excess scattering in the skin. **b:** The hair cross section is visible (bright region in lower center) after the hamster skin was put in glycerol. The skin appears much darker due to a reduction in scattering.

assume that it accounts for a majority of scattering within the skin.

Effect of Glycerol on Cells and Bulk Tissue

When a cell is exposed to glycerol, it experiences an osmotic stress, which causes intracellular water to travel along the concentration gradient and leave the cell [23]. At the same time, glycerol will passively diffuse into the cell. The diffusion coefficient of glycerol is much smaller than that of water, so it will travel into the cell at a much slower rate, causing the cell to shrink in size. At the point when equilibrium is reached, water will begin reentering the cell, while glycerol is diffused out, causing the cell to expand to its original shape. The point of minimum size for a single cell occurs on the order of a few minutes at body temperature. In bulk tissue, the diffusion time of the hyperosmotic agent to diffuse into deeply buried structures is much longer. For bulk skin, the hyperosmotic nature of glycerol draws interstitial water out of the tissue and, at a slower rate, will replace the water and salts of the ground substance. It should be noted that the present study looked at the effect of glycerol over a short period of time (~20 min), which could very well be shorter than the time required for water to reenter all of the cells in the tissue. We did not note a return of the tissue to a turbid media within the time frame of our experiments. Future work will investigate whether this return takes place.

Mechanism of Reduced Scattering

We believe that the primary mechanism of reduced scattering reported in the present study is index matching between the ground substance and the collagen fibrils. The next important factor is hypothesized to be dehydration. We do not know how much of the reduction is due to a closely index-matched environment between the collagen fibers and the ground substance, or how much is due to dehydration. The two may be related. The contribution by other skin constituents is assumed to be small, but these constituents do contribute to the overall scattering.

Dehydration of tissue has been shown to cause some reduction in scattering. In particular, one study that investigated the effect of dehydration on aorta has described a decrease in scatter-

ing over a period of 48 hr [8]. Because glycerol has a higher refractive index than the interstitial substance, its diffusion into tissue creates a more closely index-matched environment between the collagen and the surrounding interstitial space, which results in less scattering at ground substance and at the cellular and collagen interfaces. In addition, penetration of glycerol into cells leads to an additional decrease in mismatch between the intercellular matrix and the cytoplasm. This decrease in mismatch may lead to an increase in scattering because of a mismatch with intracellular organelles. The effect that glycerol has on these components and the degree of overall scattering of bulk tissue by these components are not known.

Although the effect of glycerol on cell size is known, we have not determined its effect on the diameter and spacing of collagen fiber. Since glycerol penetrates the tissue at a much slower rate than the outward diffusion of water, collagen fibrils may become more closely packed. This will reduce multiple scattering if the packed fibers act as a single scatterer.

The scattering associated with cells in the skin, such as keratinocytes and fibroblasts, is a function of wavelength, size, and refractive index. For some period of time, the application of glycerol causes cells in the skin to shrink. A reduction in diameter with no change in refractive index or volume fraction would result in a decrease in scattering contribution from these cells [24]. Although extracellular water loss reduces the overall volume with the introduction of the osmolyte, we assume cellular volume fraction has not increased. We have not established what the change in cellular volume fraction is with the introduction of the osmolyte to skin or the concentration of the osmolyte in the cells. A study by Liu et al. [24] investigated the change in optical properties of tissue with variations in cell size, refractive index mismatch between the extracellular and intracellular spaces, and cellular volume fraction.

Assuming that the reduced scattering coefficient is proportional to λ^{-n}, the value of n estimated from Figure 3 for native skin is 1.12. This value is in agreement with values found for aorta [8]. Our results showed a decrease in the value of n for increased time in glycerol, opposite of what was found for dehydrated aorta. This finding may indicate that there is more than simple dehydration occurring. Further analysis is required to quantify the mathematics involved in skin scattering, taking into account the transport of the osmolyte in the extracellular matrix and into cells and the effects on local scattering.

Subdermal Injection

A marked clearing effect through the skin of an in vivo model occurred within minutes of application of glycerol subdermally. Vasculature not visible in the native skin was evident 25 min after the injection of glycerol. The possibility that glycerol causes some vasodilation has not been completely eliminated. However, experiments in which acetylcholine, a peripheral vasodilator, was subdermally injected have failed to produce the effects seen with glycerol. Also, in vivo experiments have been performed with a resolution target under the skin of an anesthetized hamster (not shown), which show a definite increase in light transmission, causing resolution bars and vessels not seen through native skin to become clearly visible with the application of glycerol.

OCT

The potential of the method to enhance penetration depth in imaging has been shown for the specific case of OCT. The results demonstrate that the glycerol reduced excessive scattering in the tissue enough to image an underlying area that was previously not visible. However, because the source of contrast in OCT is primarily due to changes in the index of refraction, some layers are no longer visible in the more index-matched environment. Dark brown human scalp hairs have a high refractive index (1.58) [25], which is the reason the hair is visible in the index-matched tissue.

Stratum Corneum as a Barrier

It was necessary to apply glycerol to the dermal side of both in vitro and in vivo skin samples because the penetration of glycerol through the epidermis was quite limited due to the protective nature of the stratum corneum. For the method to work while applying an index-matching agent topically, efficient delivery of the agent to the dermis is required. Some possibilities for efficient delivery are the removal of the epidermis by laser resurfacing a small area or by a transdermal drug delivery device. Recent publications have demonstrated a transdermal drug delivery technique that uses pressure shock waves to transport molecules across the skin [26]. Another possibility is the use of a microporation technique that uses an excimer laser to make small holes through the epidermis. Glue stripping has been used to re-

move the epidermis from skin [27] and has been suggested for this application; however, the simple use of tape to remove the stratum corneum was not successful in the present research.

CONCLUSION

We have demonstrated that a hyperosmotic agent of refractive index similar to that of skin constituents causes a marked reduction in scattering in the skin. In addition, experiments involving OCT show that the use of an osmotically active, index-matching fluid such as glycerol increases depth of penetration, which may be useful for imaging modalities. Although glycerol is biocompatible, it does irritate tissue and may not be the agent of choice for an in vivo procedure. Optical property changes are not given in this article for in vivo clearing of skin; images comparing the in vivo and in vitro cases indicate that the results of optical property measurements will be similar. We have not determined a mathematical relation among tissue thickness, reduced scattering, and absorption with visibility. Clearly, the agent drastically increases the optical depth of the tissue. A complete picture of the mechanisms that cause the reduction in scattering when the agent is applied is not fully understood at this time. The use of hyperosmotic agents provides an excellent method for studying the mechanisms of scattering in tissue. Further experiments and analysis of the scattering mechanisms involved are required.

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